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Lack of association between Caucasian lung cancer risk and O^6-methylguanine-DNA methyltransferase-codon 178 genetic polymorphism

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Summary The formation of DNA adducts is thought to be a critical step for the induction of chemically induced cancer. O^6-Methylguanine-DNA methyltransferase (MGMT) is a ubiquitously expressed enzyme that repairs DNA adducts formed by alkylating carcinogens. Thus, genetic polymorphisms of the *MGMT* that could result in differences in MGMT activity are potential risk factors for cancer. In the present study, we established a convenient and reliable genotyping method for the *MGMT* codon 178 polymorphism, a Lys (AAG) to Arg (AGG) substitution, using restriction fragment length polymorphism (RFLP), and studied differences in the distribution of this polymorphism in 92 Caucasian lung cancer patients and 85 controls. Frequencies of the ''A'' and ''G'' alleles (*MGMT* codon 178, AAG and AGG, respectively) were 0.91 and 0.09, respectively. The genetic polymorphism of the *MGMT* codon 178 was linked with that of the *MGMT* codon 143 ($P < 0.05$). The distribution of the *MGMT* codon 178 genetic polymorphism was not significantly different between lung cancer patients and controls. Thus, our study suggests that the *MGMT* codon 178 (and possibly 143) polymorphisms do not appear to markedly affect lung cancer risk for this population. In addition, we found an apparent 10 bp-deletion in the intron before exon 5 by DNA sequencing. Because this ''deletion'' was observed in all sequenced samples ($N = 20$), the previously reported human (Caucasian) *MGMT* gene sequence should be revised to exclude this 10 bp segment.

1. Introduction

Living organisms possess DNA repair enzymes [1,2] that mitigate against the effects of DNA damage that occur as a consequence of normal life.

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O^6-Methylguanine-DNA methyltransferase (MGMT) is one such enzyme that can repair damage due to the alkylation of DNA [3]. The formation of DNA adducts is thought to be a critical step for induction of chemically induced cancer as a result of nitorosamine exposure [5,6] and for the action of alkylating chemotherapeutic agents [3]. Consequently, individual differences in MGMT expression have been the topic of attention in both cancer prevention and cancer therapy [3,4]. In the case of lung cancer, circumstantial evidence suggests that tobacco-derived carcinogen-DNA adducts, such as O^6-methylguanine, are responsible, in part, for the development of the disease [3,4]. Thus, individual differences in MGMT-dependent repair capacity could contribute to individual variability in lung cancer risk. For example, human *MGMT* transgenic mice show reduced lung tumorigenesis [7] compared to *MGMT* knockout mice, and *MGMT* knockout mice are susceptible to large numbers of lung adenomas [8]. In addition, MGMT expression was found to be different in smokers and nonsmokers with non-small cell lung carcinomas [9].

Several genetic polymorphisms in the *MGMT* have been reported [10—16]. Of these, a single nucleotide polymorphism (SNP) at codon 143 (exon 5) of *MGMT*, Ile (ATC) to Val (GTC), has been reported to be associated with susceptibility to esophageal and lung cancer [15,16]. The importance of amino acid residue 143 may be that it is near to the conserved alkyl group acceptor Cys 145. A second SNP in codon 178, Lys (AAG) to Arg (AGG), has been shown to be in linkage disequilibrium with the SNP at codon 143 [15,17]. However, the consistency of linkage between the two SNPs and the significance of the codon 178 polymorphism for cancer risk have not been widely investigated. The reported methods for genotyping of codon 143 [15,17] or 178 [15,17] polymorphism require the use of radioisotopes; thus, a more convenient and rapid genotyping method is desirable. For this purpose, we established a simple genotyping method for the *MGMT* codon 178 genetic polymorphism using restriction fragment length polymorphism (RFLP). We further investigated the linkage between the two SNPs at the *MGMT* codon 143 and 178 and the differences in distribution of this genetic polymorphism in lung cancer patients and controls.

2. Materials and methods

2.1. Subjects and samples

The study subjects were 216 Caucasian lung cancer patients and controls taken from a study conducted at the National Naval Medical Center (NNMC), Bethesda, MD and 10 Caucasian lung cancer patients from the Central Arkansas health Administration (CAVHS) Hospital, Little Rock, AR. All study subjects gave signed informed consent. Details of the NNMC population have been published previously [18]: In detail, patients undergoing evaluation for possible lung cancer were identified and recruited from all departments that manage lung cancer patients in NNMC (1988—1992). Cases were included in the analysis only after their histological diagnosis of lung cancer. Controls were recruited from outpatients with scheduled appoints in the Urology and orthopedic Surgery clinics at the NNMC and matched to cases by 5-year age group, sex and race (Caucasians). Blood and lung tissue samples were collected from the NNMC and CAVHS, respectively (mean age, 59.1 years old; male, 65.9%; never smokers, 26%; former and current smokers, 74%). Statistical analyses for lung cancer risk were restricted to a subset of NNMC subjects for whom physical and lifestyle information was available ($N = 177$).

DNA was isolated from the peripheral blood or normal and cancerous lung tissues of subjects using the phenol/chloroform extraction after proteinase K digestion [19].

2.2. MGMT genotyping

To determine the *MGMT* codon 178 genotypes, we developed a PCR-RFLP method with an artificially mismatched forward primer, MGMT178f, 5′-atgaaggccaccggttg**a**gg-3′ (the bold letter indicates an artificially introduced mismatch) and a reverse primer, MGMTr, 5′-cccaggacactgccacttcct-3′. PCR conditions were as follows: 50 μl of PCR mixture contained 50 mM KCl, 10 mM Tris—HCl (pH 9.0), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, 100 ng of DNA, and 2.5 U of Taq DNA polymerase (Promega, Madison, WI). The PCR reaction was started at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s; final extension was performed at 72 °C for 7 min.

Amplified PCR products were digested with *BseR1* (New England Biolabs Inc., Beverly, MA). The 20 μl of digestion mixtures containing 8—17.5 μl of the PCR product, and 2 U of *BseR1* were incubated for 5 h at 37 °C. Five microliters of each digested sample was loaded on a 1.8% Metaphor agarose gel (FMC, Rockland, ME) for electrophoresis, and visualized by ethidium bromide staining under an ultraviolet light in a MultiImage™ II Light cabinet and an Impression® 5VXL monitor-connected to a Chemoimager™ 4400 (Alpha Innotech Corporation, San Leandro, CA). We refer to the alleles as ''A'' or

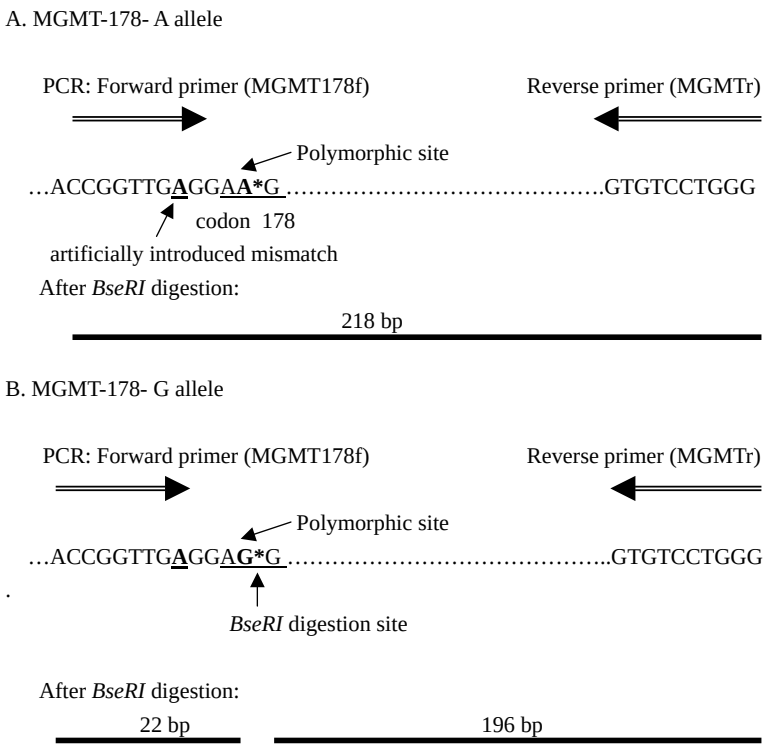

Fig. 1 Primer design for the PCR-RFLP for MGMT codon 178. Gene-specific primers were used to amplify *MGMT* exon 5 in the polymorphic region. The forward primer (MGMT178f) contains a mismatch that generates a *BseR1* restriction enzyme site specific for the codon 178 G variant.

''G'' depending on the presence of A**A**G or A**G**G of codon 178, respectively. The G allele was digested by *BseR1* into 22 and 196 bp fragments, whereas the A allele lacked the *BseR1* restriction site (Fig. 1).

2.3. DNA sequencing analysis

In order to confirm the specificity of the RFLP for the MGMT 178 polymorphism, we sequenced PCR products derived from 20 subjects who were determined by PCR-RFLP to be heterozygous A/G ($N = 19$) or homozygous G/G type ($N = 1$). The forward primer (SF) is upstream of the PCR-RFLP primer and the resultant PCR products included both polymorphic sites of exon 5 (Fig. 2). Thus, we were able to observe linkage disequilibrium between SNPs at codons 143 and 178 for these samples. PCR conditions were as stated above. Dye terminator DNA sequencing was performed both 5′—3′ and 3′—5′, using ''BigDye'' terminators (PE Biosystems, Foster City, CA), the primer SF or MGMTr, and a PRISM 310 Genetic Analyzer (PE Biosystems). PCR products were purified before dye-termination using Qiaquick columns (Qiagen, Chatsworth, CA)

Fig. 2 Relationship of PCR-RFLP and sequencing primers for *MGMT* exon 5. *MGMT* exon 5 was amplified using the gene-specific primer SF (downstream of the primer MGMT178f) and the primer MGMTr (common to both PCR and sequencing analyses). Exonic and intronic sequences are shown by large and, small characters, respectively: the previously reported 10 bp sequence shown in bold and in parentheses was absent, sequencing both 5′—3′ and 3′—5′.

and dye-terminated products were purified using Centri-Sep columns (Princeton separations, Adelphia, NJ).

2.4. Statistical analysis

The relationship of genotype to lung cancer was assessed by odds ratio (OR) approximation of the relative risk, and the 95% confidence interval (CI). Both were obtained from logistic regression models from within the BMDP statistical analysis program [18].

3. Results

3.1. Distribution of codon 178 polymorphism

Fig. 3 shows the results of *MGMT* codon 178 genotyping using RFLP. Table 1 shows the distributions of the A and G alleles in the 226 samples. Frequencies of the A and the G allele were 0.91 and 0.09, respectively and follows the Hardy—Weinberg equilibrium ($P = 0.51$). After genotyping, we selected 19 subjects of A/G type and one subject of G/G type

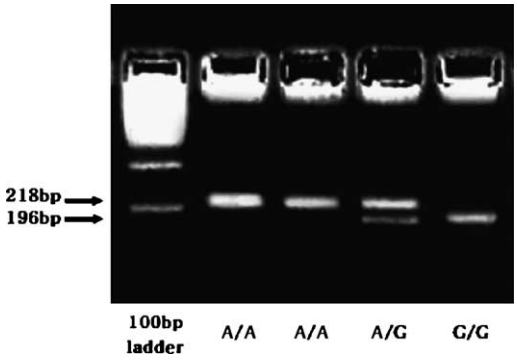

Fig. 3 Examples of PCR-RFLP analysis of *MGMT* codon 178 polymorphism. PCR products (see Fig. 1) were digested with excess *BseR1* and the products resolved on a 1.8% agarose gel with ethidium bromide staining. The tracks correspond to molecular weight markers, homozygotes of A allele (A/A), homozygotes of G allele (G/G) or heterozygotes of A and G allele (A/G).

Table 1 Distribution of A and G alleles of MGMT in all subjects

Genotype	Number of subjects (%)
A/A	186 (82.3)
A/G	39 (17.3)
G/G	1 (0.4)
Total	226 (100.0)

Table 2 Linkage between SNPs at the MGMT codons 143 and 178 in selected subjects[a,b]

	Codon 178	
	A/G	G/G
Codon 143 A/G	17 (89.5)	0 (0.0)
Codon 143 G/G	2 (10.5)	1 (100.0)
Total	19 (100.0)	1 (100.0)

[a] Data show number of subjects (%).
[b] Fisher's exact test, $P < 0.05$.

to clarify the nature of the link between SNPs at codons 143 and 178. We found that codon 143 mutation was linked with codon 178 mutation (Table 2). We also found that the genotyping results of *MGMT* codon 178 by the RFLP were precisely the same as those obtained by sequencing. Thus, we believe that our RFLP method is a reliable way of determining genetic polymorphism at *MGMT* codon 178.

3.2. Sequence of MGMT

During the above sequencing *MGMT*, we found a 10 bp-deletion (Fig. 2) in the intron before exon 5. Thus, the sequence of human *MGMT* reported by other researchers [20] should be revised to exclude this 10 bp segment in Caucasians.

3.3. Effects of MGMT codon 178 genetic polymorphism on lung cancer susceptibility

To determine whether the *MGMT* codon 178 genetic polymorphism affects lung cancer susceptibility, we investigated the distribution of this polymorphism in lung cancer patients and controls, from whom we had obtained age, sex, smoking, and education information. As a result (Table 3), there were no differences in incidences of lung cancer due to the *MGMT* 178 polymorphism ($\chi^2 = 0.23$, $P = 0.63$). When we categorized cases according to histological diagnosis, such as squamous carcinoma, small cell carcinoma, etc., each category included too small a number of subjects to be statistically meaningful, and therefore, we did not investigate the effects of the codon 178 polymorphism on lung cancer histology.

4. Discussion

Some of genetic polymorphisms in metabolic or DNA repair enzymes have shown to affect lung cancer susceptibility [21]. The *MGMT*-codon 143 polymorphism among them was reported to be

Table 3 Lack of association between lung cancer risk and the MGMT codon 178 polymorphism

Genotype	Cases	Controls	OR[a]	95% CI
A/A	72 (78.3%)	69 (81.2%)	1.00	
A/G	20 (21.7%)	16 (18.8%)	1.19	0.46—1.93
Total	92 (100.0%)	85 (100.0%)		

[a] Adjusted for age, sex, smoking, and education.

associated with lung cancer [16] in a mixed population with African—Americans ($N = 81$) and Caucasians ($N = 55$). In addition, the *MGMT*-codon 178 polymorphism was reported to be linked with the *MGMT*-codon 143 polymorphism [15,17]. Considering ethnical differences and sample size, we investigated effects of the *MGMT* polymorphism on lung cancer incidence in an age and sex matched, and enlarged Caucasian population ($N = 177$) and found that the *MGMT*-codon 178 polymorphism was linked with the *MGMT*-codon 143 polymorphism (Table 2) and that the *MGMT*-codon 178 polymorphism was not associated with lung cancer risk (Table 3). In addition, the frequency of the rare allele (G allele) of the *MGMT*-codon 178 polymorphism in this study was not much different from those in others' Caucasian studies (0.09 versus 0.11—0.13, respectively [17,22]). Due to small differences in the allele frequencies between cases and controls (Table 3), a big sample size, at least, $N = 1763$ in each case and control group, is required for the statistical power with 60% to support the above lack association. However, it is very hard to collect over 3000 subjects. Therefore, to prevent false negative results without increasing sample size, studies, which are based on small sample sizes, should be done in various laboratories and subjects to bring out reproducible results. Therefore, the present study is thought to be a required study for clarification of effects of the *MGMT*-codon 143/178 genetic polymorphisms on Caucasian lung cancer.

Recently, the *MGMT* various SNPs including the codon 143/178 polymorphism were investigated to study effects of the MGMT polymorphisms on familial melanoma risk and response to chemotherapeutics [17,23]. However, no statistically significant differences were seen between cases and controls for any of the *MGMT* SNPs [17]. On the other hand, there was no evidence that these variants decreased the *MGMT* DNA repair activity compared to the wild-type protein [23]: In addition, there was no significant correlation between any of the *MGMT* polymorphisms and clinical response to chemotherapy, although an indication of a lower response rate in patients with SNPs in exon 5 (the codon 143/178 polymorphism) was obtained. Thus, *MGMT* expression appears to be more related to response to chemotherapy than *MGMT* polymorphisms in patients with metastatic melanoma. We also studied association between Caucasian lung cancer risk and phenotypical variation in the *MGMT* in the present study: Using Western blotting, we tried to quantify MGMT protein expression in the CAVHS lung tissues ($N = 10$) to study the effects of the *MGMT* codon 178 polymorphism on MGMT protein expression. However, we found that the presence of MGMT is low in the human lung compared to other organs, such as the liver, and we were unable to detect MGMT protein expression in the normal and cancerous lung tissues by Western blotting (data not shown). Therefore, it is possible that the low level of expression of MGMT in lung results in lack of a significant role for the protein in pulmonary DNA repair and consequently low impact of the polymorphism on lung cancer risk. Esteller et al. [24] suggested that the loss of *MGMT* expression is due to silencing of the gene by methylation of discrete regions of the CpG islands, and rarely due to deletion, mutation, or rearrangement: they found aberrant methylation of *MGMT* in 25% of non-small cell lung carcinomas. Thus, the effects of epigenetic or environmental factors on MGMT expression may be greater than *MGMT* genetic polymorphisms.

In conclusion, the frequencies of A and G alleles (as occupants of MGMT codon 178, AAG and AGG, respectively) in 226 Caucasians were 0.91 and 0.09, respectively. The genetic polymorphism of the *MGMT* codon 178 was linked with that of the *MGMT* codon 143. The distribution of the *MGMT* codon 178 polymorphism was not significantly different in lung cancer patients and controls in the Caucasian population. Our study suggests that the *MGMT* codon 143/178 genetic polymorphism dose not affect Caucasian lung cancer risk.

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